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Identification and functional characterizations of a novel *TRIF* gene from grass carp (*Ctenopharyngodon idella*)

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ABSTRACT

Toll/interleukin-1 receptor (TIR) domain containing adapter inducing interferon-β (TRIF) is an adapter in responding to activation of some toll-like receptors (TLRs), which provides early clearance of viral and bacterial pathogens. Here we identified and characterized a full-length genomic sequence of *TRIF* gene from grass carp *Ctenopharyngodon idella* (designated as *CiTRIF*). *CiTRIF* genomic sequence consists of 3534 base pairs (bp), containing 5′ flank sequence (496 bp) and unique intron (815 bp). The full-length cDNA sequence is 2241 bp, including 5′ untranslated region (UTR) of 352 bp, 3′ UTR of 209 bp, and an open reading frame of 1680 bp encoding a polypeptide of 559 amino acids with an estimated molecular weight of 62.643 kDa and a predicted isoelectric point of 5.71. The deduced amino acid sequence just contains TIR domain, and is most similar to the zebrafish (*Danio rerio*) TRIF sequence with an identity of 64%. *CiTRIF* exhibits sequence divergence from its orthologs. Promoter region was predicted and promoter activity was verified. mRNA expression of *CiTRIF* gene is widespread in 15 tissues investigated, highly in foregut and skin physiological immune barrier. The transcripts of *CiTRIF* were significantly and rapidly induced in spleen and head kidney tissues at early stage post grass carp reovirus (GCRV) challenge. The modulations are significant but mild in CIK (*C. idella* kidney) cells post GCRV infection or poly(I:C) stimulation. The over-expression vector was constructed and transfected into CIK cell line to get stably expressing recombinant proteins. In *CiTRIF* transfected cells, mRNA expressions of *CiTRIF*, *CiRIG-I*, *CiIRF7* and *CiIFN-I* were up-regulated. After GCRV infection, the transcripts of *CiTRIF*, *CiRIG-I*, *CiIRF7* and *CiIFN-I* fell a little bit after a rapidly and strongly rise. In *CiTRIF* over-expression cells, virus load and titer were significantly lower than those in controls post GCRV challenge, and virus replication was inhibited obviously. The results indicate that the novel *TRIF* gene from grass carp plays important roles in modulating antiviral innate immune responses, and serve the further functional studies on *TRIF* gene in teleosts and immune evolution.

1. Introduction

The innate immune response is the first line of defense against infectious diseases. The innate immunity is the primary defense mechanism among invertebrates, and plays a key role in vertebrate animals as well, particularly in lower vertebrates, such as fishes, whose adaptive immunity is relatively less developed. The innate system recognizes non-self and danger signals by a limited number of germ-line encoded pattern recognition receptors (PRRs). Three major PRR families, toll-like receptors (TLRs), retinoic acid inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide binding oligomerization domain (NOD)-like receptors (NLRs), recognize different but overlapping pathogen associated molecular patterns (PAMPs) (Kawai and Akira, 2009). TLRs are the earliest characterized and the most extensively studied PRRs (Zhang et al., 2013).

Upon activation of TLRs not only initiates innate immune defenses, but also regulates specific adaptive immunity, bridging innate and adaptive immunity (Aderem and Ulevitch, 2000). Thirteen TLR members and five adaptor molecules have been identified in mammals (Kenny and O'Neill, 2008). The adaptors include myeloid differentiation factor 88 (MyD88), toll/interleukin-1 receptor (TIR) domain containing adapter inducing interferon-β (TRIF), TIR domain-containing adaptor protein (TIRAP or MAL), TRIF-related adaptor molecule (TRAM or TICAM2), and sterile α and armadillo motif-containing protein (SARM) (Kenny and O'Neill, 2008).

TRIF, also known as TICAM-1 (TIR domain-containing adapter molecule 1), plays an essential role in TLR3- and TLR4-mediated interferon-β (IFN-β) activation through interferon regulatory factor 3/7 (IRF3/7)-dependent mechanism (Doyle et al., 2003; Fitzgerald

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et al., 2003). In piscine cells, both fugu *Takifugu rubripes* TLR3 (fgTLR3) and fgTLR22 employ the TRIF adaptor to induce IFN responses upon virus infection (Oshiumi et al., 2003; Matsuo et al., 2008). TRIF provides early clearance of viral and bacterial pathogens (Hyun et al., 2013). Mammalian TRIF consists of N-terminal region, TIR-domain and C-terminal region. The N-terminal region harbors tumor necrosis factor (TNF) receptor-associated factor (TRAF) family proteins and forms a complex containing IRF3-activating kinase TBK1 (TANK-binding kinase-1), which is crucial for activating IFN-β (Sharma et al., 2003; Oganesyan et al., 2006). TIR domain contains three highly conserved regions (Box 1-3), which is an intracellular signaling domain that mediates protein-protein interactions between the TLRs and signal-transduction components with TIR domain (Fan et al., 2008; Lu et al., 2008). The C-terminal region recruits receptor-interacting protein-1 (RIP-1), and this event is followed by the activation of other effectors (Meylan et al., 2004).

Upon activation by PAMPs, TLRs interact with TRIF to activate TBK1 and phosphorylate IRF3/7 (Fitzgerald et al., 2003; Yamamoto et al., 2003), thus signals are transmitted into nucleus, where the transcription of *IFN-β* and other IFN-regulated genes are activated (Honda et al., 2005).

To date, TRIF homologs have been identified in several vertebrates. However, in teleosts, complete coding sequence of *TRIF* is just reported in channel catfish (*Ictalurus punctatus*) (Baoprasertkul et al., 2006), zebrafish (*Danio rerio*) (Fan et al., 2008) and *T. rubripes* (accession No., AB197917) in GenBank. Piscine TRIF plays an important role in immune response to bacteria (Baoprasertkul et al., 2006).

Grass carp (*Ctenopharyngodon idella*) is one of the most important freshwater aquaculture species in China. However, grass carp reovirus (GCRV) causes serious losses to the grass carp farming industry, resulting in a high mortality rate and an enormous economic loss to the aquaculture industry (Su et al., 2010). Better understanding of the immune defense mechanisms of grass carp may contribute to the development of management strategies for disease control and long term sustainability of grass carp farming. To explore the antiviral immune responses and functions of TRIF in grass carp, the full-length cDNA and genomic DNA sequences of *TRIF* (*CiTRIF*) gene were identified and characterized from grass carp. The promoter activity was investigated. The mRNA expression profiles were investigated post-GCRV challenge *in vivo* and *in vitro*. Further, the immune functions were carried out in cells over-expressing *CiTRIF*. The results will facilitate functional studies on *TRIF* gene in teleosts and serve the immunological control of viral diseases.

2. Materials and methods

2.1. Fish, viral challenge and sample collection

Grass carp, GCRV challenge and tissue samples were prepared as previous report (Yang et al., 2013).

2.2. Cells, immune stimulations and sample preparation

C. idella kidney (CIK) cells, GCRV or poly(I:C) stimulations and sample preparations were performed as previous study (Su et al., 2012).

2.3. Cloning of the full-length cDNA of CiTRIF

The first fragment of *CiTRIF* gene was obtained by screening a grass carp genomic library. The sequence was confirmed by PCR-amplification with TF621 and TR622 primers (Table S1). The PCR product was ligated, transformed, plated, screened and sequenced as previous study (Su et al., 2010). After sequence analysis, it locates in an exon of TRIF gene. To identify the 5′ and 3′ unknown sequences, rapid amplification of cDNA ends (RACE) was performed using the 5′ RACE system (Invitrogen) and BD SMART™ RACE cDNA amplification kit (BD Biosciences Clontech). The first strand cDNA synthesis and RACE were conducted on gill-derived RNA. To obtain the 3′ unknown region, primer pairs TF652/UPM and TF653/NUP (Table S1) were used for the primary PCR and the nested PCR respectively. Similarly, the 5′ end of *CiTRIF* gene was also obtained by nested PCR, using primer pairs TR654/AAP and TR655/AUAP (Table S1). All the PCR products were prepared and sequenced as above. The complete cDNA sequence of *CiTRIF* was assembled by overlapping the three fragments and confirmed by sequencing the PCR product amplified by primers TF713a and TR714a (Table S1) within the predicted 5′ and 3′ untranslated regions (UTR), respectively.

2.4. Detecting the intron(s) and identifying the 5′ flank sequence of CiTRIF

Genomic DNA was extracted from grass carp spleen. Three pairs of *CiTRIF* primers (Table S1), covering the full-length cDNA sequence, were employed for genomic sequence study. The sequencing result was aligned with the cDNA sequence and the genomic structure of *CiTRIF* was obtained. Genomic organizations of other representative *TRIFs* were got from GenBank.

The 5′-flanking sequence of *CiTRIF* gene was PCR-amplified from genomic DNA according to the protocol of Genome Walker kit (Clontech). The first round of PCR extension was performed with primers AP1/TR730 (Table S1). The second round of PCR amplification was carried out with primers AP2/TR731 (Table S1). The PCR product was sequenced as above and overlapped with the above sequence. The full-length genomic sequence of *CiTRIF* gene was obtained.

2.5. Sequence analysis

The searches for nucleotide and protein sequence similarities were conducted with BLAST program (http://www.ncbi.nlm.nih.gov/blast). The promoter region was predicted by WWW Promoter Scan software (http://www-bimas.cit.nih.gov/molbio/proscan/). The CpG island was predicted by online software (http://www.urogene.org/methprimer/). Simple Sequence Repeat (SSR) was searched by SSRHunter Tool (http://en.bio-soft.net/dna/SSRHunter.html). The deduced amino acid sequence was analyzed with the Expert Protein Analysis System (http://www.expasy.org/) and the Sequence Manipulation Suite programs (http://www.bioinformatics.org/sms/). The protein domain was predicted by Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/) and putative Conserved Domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/docs/cdd_search.html). Phylogenetic tree was constructed based on the deduced amino acid sequences by the neighbor-joining (NJ) algorithm embedded in Mega 5.1 program.

2.6. Exploring the promoter activity

For detecting the promoter activity, a DNA fragment containing the 5′-flanking sequence was amplified with primers TF749c and TR750a (Table S1), digested with *Bgl*II/*Bam*HI enzymes and then ligated to the pEGFP vector, a promoterless report vector, which was obtained from pCMV-EGFP (Clontech). The recombinant vector was designated as pTRIF-EGFP and the 5′-flanking fragment of *CiTRIF* located at upstream of *EGFP* gene. CIK cells (2×10^5 cells/ml) were transfected in 24-well plates with 0.5 μg of purified